ABSTRACT

Title of thesis: MODE OF BIRTH AND VAGINAL MICROBIOTA IN REPRODUCTIVE-AGE WOMEN

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It is well documented that women with robust, *Lactobacillus*-dominated vaginal microbiotas are less likely to develop a range of adverse reproductive health outcomes. The birthing process is thought to be a critical event in the initial seeding, or colonization, of the human microbiome, and the transfer of microbiota from mother to baby during delivery is associated with long-term health. We recruited 88 adult women to a cross-sectional study to evaluate the relationship between their vaginal microbiota and the mode of their birth (self-reported as vaginal delivery or Cesarean section (C-section)). In a multivariable analysis, women who had a less protective, low-*Lactobacillus* community state type had 3-fold increased odds of having been born via C-section, indicating that C-section is related to vaginal dysbiosis in adulthood (adjusted OR: 3.73, p=0.01, 95% CI: 1.08-12.77). Although the cross-sectional analysis does not account for fluctuations in microbial composition, the significant point estimate suggests that birth mode may play a role in vaginal seeding and colonization outcomes in adult women.

MODE OF BIRTH AND VAGINAL MICROBIOTA IN REPRODUCTIVE-AGE WOMEN

By

Christina Stennett

Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, College Park in partial fulfillment
of the requirements for the degree of
Master of Public Health in Epidemiology
2017

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CHAPTER I: INTRODUCTION

The human microbiome plays an essential role in sustaining health and protecting against infections in multiple body sites [1]. The bacteria, archaea, and other eukaryotes that compose the microbiome have specific immunologic and homeostatic functions, and certain variances or disruptions in the microbiome have been associated with the development of diseases such as asthma, autism, and obesity [2-4].

The birthing process is an early and critical event in the seeding, or colonization, of the baby's microbiome, which can impact child health [5]. The mode of one's birth (i.e., vaginal delivery versus Cesarean section (C-section)) is a key indicator of exposure. Neonates born vaginally are exposed primarily to the mother's vaginal bacterial environment and, to a lesser degree, bacteria gained from contact with the mother's skin and other sources. There is strong evidence that babies born vaginally are more likely to be colonized by anaerobic bacteria, such as *Escherichia coli*, *Staphylococcus*, and *Streptococcus*, which promote a healthy gastrointestinal (GI) environment [6]. However, 32.0% of U.S. births in 2015 were C-section deliveries [7]. These babies tend to harbor bacteria across all body habitats that are more similar to the skin communities of the mother [8]. Although research by Azad *et al.* suggests that these differences in microbial composition in the gut due to birth mode can be seen at least 4 months post-delivery, it remains unknown how long these characteristics persist past infancy [9]. Studies have shown that babies born by C-section are more likely to develop celiac disease, diabetes mellitus, and asthma in early childhood [10-12]. These disease outcomes have been associated with deviations in the development of the gut microbiota, with observed decreases in overall bacterial diversity and abundance of harmful bacterial taxa [13].

These findings indicate that delivery mode and early microbial colonization may not only be associated with, but also have a causal relationship, in the development of diseases related to dysbiosis of the gut microbiome.

Despite considerable evidence to suggest the persistent influence of C-section delivery on the gut microbiota, there has been little attention given to the effect of birth mode on other body sites. In addition, the available research has also been limited to outcomes in infants and children. The goal of this thesis is to investigate potential associations between a woman's mode of birth and her vaginal microbiota in adulthood. A vaginal microbiota dominant in *Lactobacillus spp.* is commonly viewed as "optimal," and numerous studies have demonstrated that women with *Lactobacillus*-dominated vaginal microbiota are less likely to develop a range of reproductive health outcomes, including bacterial vaginosis (BV), sexually transmitted infections (STIs), and cervical cancer [14-16]. As few studies explore how a healthy vaginal microbiome is initially colonized, this research will contribute to the understanding of how birth mode relates to bacterial composition in adulthood.

LITERATURE REVIEW

The microbiota in sites throughout the body differ in terms of the types, diversity, and relative abundance of the bacteria that are likely to thrive. Certain body sites, such as the skin and GI tract, usually benefit from richly diverse bacterial environments. In these body habitats, highly-diverse communities tend to be more stable and are associated with better health outcomes [17]. In contrast, vaginal communities tend to be more homogenous and are usually dominated by one or more species of *Lactobacillus* [18].

Most *Lactobacillus* spp. are protective for the vaginal environment, primarily by producing lactic acid, which lowers the regional pH and acts as an antimicrobial against pathogens [19, 20]. Without these *Lactobacillus* and lactic acid available, women are at greater risk for STIs such as gonorrhea, chlamydia, and HIV upon exposure, as well as the development of BV [21, 22]. Vaginal *Lactobacillus* spp. may also help to restrict the growth of pathogens by producing bacteriocins (bactericidal proteinaceous molecules) [23-27], antagonistic bacteriocin-like substances [28], and biosurfactants [29], and through their ability to adhere to mucus, which enable them to form a barrier against pathogens [30] and disrupt biofilms [31]. Lactic acid also appears to control overgrowth of bacteria by disrupting the integrity of some bacterial cell membranes [32-37]. In addition, there is evidence that lactobacilli influence the ability of *Trichomonas vaginalis* to adhere to host cells, thereby altering the virulence of this parasite [38]. Overall, *Lactobacillus* spp. provide multifaceted protection against pathogenic organisms and is our outcome of interest.

Vaginal bacterial communities can be broadly clustered into five groups or "community state types" (CSTs) based on their diversity and relative abundance of bacteria [18]. Four CSTs are dominated by *Lactobacillus*, *L. crispatus* (CST I), *L. gasseri* (CST II), *L. iners* (CST III), or *L. jensenii* (CST V). We hypothesize that there are functional differences among these species. In one study of adult women by Verstraelen *et al.*, *L. crispatus*-dominated profiles promoted the stability of the normal vaginal environment, and *L. iners* and *L. gasseri* were more highly associated with prevalence of dysbiotic, or unbalanced, vaginal microbiota [39]. Gajer and Brotman *et al.* also found that *L. iners*-dominated CST III was associated with both stable and fluctuating patterns

in and out of BV-associated states, but *L. gasseri*-dominated profiles were remarkably stable [40]. CST IV is unique in that it is a low-*Lactobacillus* state which is characterized by the abundance of BV-associated anaerobic organisms, including *Gardnerella, Megasphera, Sneathia,* and *Prevotella*. Yeoman *et al.* have hypothesized that several of the taxa found in abundance in CST IV may contribute to vaginal dysbiosis and BV by producing biogenic amines [41].

There is little data on how the vaginal microbiome is initially seeded. The establishment of stable vaginal microbial communities may rely on several early environmental factors. Following a vaginal birth, a neonate is covered in the vaginal bacteria from the mother, which have been shown to have an immediate effect on the short-term colonization outcomes at several body sites [5]. However, babies born by Cesarean delivery do not transit through the environment of protective vaginal bacteria. Babies born via C-section have an increased risk of childhood-onset type 1 diabetes and obesity at age 3 years, outcomes that may be related to dysbiosis in the microbiota of the GI tract [42, 43]. Longitudinal studies investigating the effect of birth mode and neonatal exposures have followed infants for 2 years or less. For example, Chu *et al*. described a cohort study of 81 mother and infant dyads in which multiple body sites, including stool, oral gingiva, nares, skin and vagina, were sampled for 6 weeks [44]. They observed minor variations in the neonates' microbiota community structure associated with C-section delivery in most body sites immediately after birth. However, while the infants' microbiota changed significantly at each body site, they reported no discernable differences in community structure or function between infants delivered vaginally or C-section at 6 weeks of age. This study did not collect infant vaginal samples to determine

how the vaginal microbiota undergoes reorganization in early life, which underscores the need for longitudinal studies assessing the seeding of bacteria in the vaginal environment.

After infancy, the vaginal microbiome undergoes significant transitions. In early childhood, girls tend to be colonized by stable aerobic, anaerobic, and enteric bacteria [45]. Important findings by Hickey *et al.* suggest that the vaginal microbiota of girls begins to resemble those of adults (typically dominated by *Lactobacillus* spp.) before menarche, while girls are still in the early and middle stages of puberty [46]. It is thought that the composition and function of the vaginal microbiota changes in puberty due to increased estrogen-stimulated glycogen production in the vaginal epithelium [47]. However, it remains unclear how girls transition and colonize adult-like vaginal microbiota, and further studies are needed to explore the influence of genetic and environmental factors. This thesis will help address this knowledge gap by investigating the environmental exposures caused by a woman's mode of birth and her bacterial colonization in adulthood.

Although the first menstrual period (menarche) signals the possibility of fertility, it is accompanied by several physiological and anatomic changes, which may impact the vaginal microbiome. Factors associated with early menarche include levels of subcutaneous fat levels, BMI, and being formula fed as an infant [48]. The mean age at menarche in the United States is approximately 12.5 years, according to studies conducted in 1994 [49, 50]. These sources also indicate a decrease of about 2 to 4 months in the average age since the 1970s. Black and Hispanic women tend to have a lower average age at menarche compared to White and Asian women, and higher relative weight in adolescence is associated with increased likelihood of early menarche [50].

Early age at menarche (younger than 11) is a critical outcome to study because it has been found to be a risk factor for asthma, insulin resistance, total number of metabolic syndrome components, and increased cardiovascular risk in adulthood [51-53].

Once an adult-like vaginal microbiota is established, a woman's CST can fluctuate over time due primarily to menstrual bleeding and changes in estrogen and progesterone levels [54]. Further, race/ethnicity, age, body mass index (BMI), hygiene practices, and sexual behavior are all associated with variances in the microbiota [1, 18, 55, 56]. Among these risk factors, race/ethnicity is most strongly linked with elevated vaginal pH and microbial composition, with Hispanic and African American women more likely to have low-*Lactobacillus* microbiota compared to Caucasian and Asian women [14]. Studies of the vaginal microbiome must take host intrinsic and extrinsic factors into account as they play a role in microbial composition and disease outcomes.

The dysbiosis in the vaginal microbiome, and its clinical syndrome bacterial vaginosis, have long been considered a nuisance factor in women's health research. There is overwhelming data that disruptions in the vaginal microbiota lead to a wide range of adverse outcomes related to women's health. Current efforts are focused on development of new therapies to restore vaginal health. Antibiotics such as metronidazole and clindamycin are the current standard for treating BV [57]. Pre- and probiotics have been suggested (inconclusively) to promote dominance by *Lactobacillus* spp. that will help stabilize the vaginal microbiota as well as fend off infection by sexually transmitted pathogens and importantly, could be antibiotic-sparing [58, 59]. Another preventive strategy may be to ensure that newborns are exposed to beneficial microbes at birth. The intention of "seeding" a C-section infant is to transfer microbes from the mother that the

infant would normally have obtained during vaginal birth. In a recently published pilot study, Dominguez-Bello and colleagues found that C-section infants who were seeded with vaginal secretions from their mother had gut, oral, and skin bacterial communities enriched in vaginal bacteria after 30 days, similar to infants born vaginally [60]. There is still controversy surrounding this practice, including transfer of pathogens such as herpes simplex virus, and the long-term risks and benefits are unknown. There is no evidence currently available from longitudinal studies showing C-section birth as being a risk factor for developing vaginal dysbiosis and related diseases at reproductive age. Although the enclosed thesis is cross-sectional and cannot account for transitions in the microbiome over the life span, it provides the impetus for further study and importantly, presents preliminary data in describing the relationship exposure to vaginal microbes at birth to vaginal health in adult women.

RESEARCH QUESTION AND SPECIFIC AIMS

The overall goal of this research is to determine whether the mode of birth is associated with vaginal microbiota composition in adulthood. I hypothesize that women born by C-section will be more likely to lack the protective vaginal *Lactobacillus* spp. or to be in a somewhat unbalanced (quasi-dysbiotic) state defined by predominance of *L. iners*. Numerous studies have documented that a low-*Lactobacillus* state creates a microenvironment in which women are more susceptible to STIs and urinary tract infections [61-63]. This research attempts to uncover birth mode as a risk factor for these adverse outcomes. The directed acyclic graph (DAG) in Figure 1 illustrates the interplay among the variables identified in the specific aims.

SPECIFIC AIM 1: To measure the association between birth mode and the presence of protective vaginal microbiota in adult women.

HYPOTHESIS: Women with a dysbiotic vaginal microbiota defined by low-*Lactobacillus* (CST IV) as well as *L. iners*-dominated (CST III) community profiles will be more likely to have been born via C-section. In contrast, women with more protective CSTs (dominated by *L. crispatus* (CST I), *L. jensenni* (CST II), or *L. gasser*i (CST V)) will be more likely to have been born via vaginal delivery.

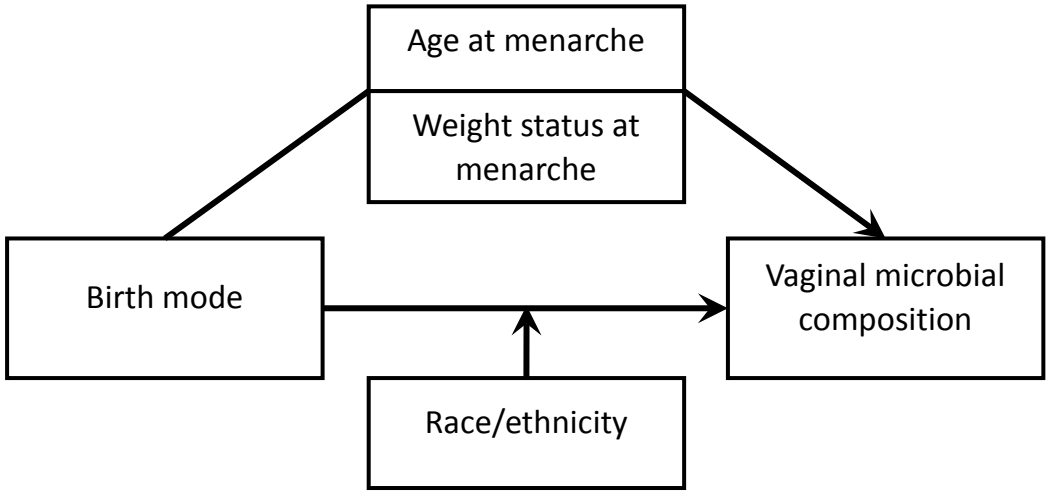
SPECIFIC AIM 2: To determine if the associations between birth mode and vaginal microbiota are modified by race/ethnicity, a major intrinsic factor known to be associated with disparities in the vaginal microbial composition and in C-section rates.

HYPOTHESIS: The magnitude of the association between birth mode and microbiota composition (CST) in adulthood will differ among racial groups.

SPECIFIC AIM 3: To test for confounding in the association between birth mode and vaginal microbiota by age and weight at menarche.

HYPOTHESIS: Younger age at menarche, as well as higher perceived weight status during adolescence, will be associated with C-section delivery and low-*Lactobacillus* vaginal states, producing a confounding effect in the model.

Figure 1: Directed acyclic graph with major variables



CHAPTER II: METHODS

OVERVIEW

The proposed cross-sectional study involved a cohort of reproductive-age women who were enrolled in the Hormonal Contraception Longitudinal (HCL) Study (described in detail in the following section). In addition to a secondary analysis of HCL Study data, primary data obtained in this study included birth mode and early childhood factors.

HORMONAL CONTRACEPTION LONGITUDINAL (HCL) STUDY

Conducted by Johns Hopkins School of Medicine researchers (PI: Khalil Ghanem, MD, PhD), the HCL Study (NIAID R01-AI089878) aims to describe how the initiation or cessation of hormonal contraceptives (HCs), including oral contraceptive pills, vaginal rings, etonogestrel contraceptive implants, and other methods, affect the vaginal microbiota and immune response in the lower genital tract.

The HCL Study recruited women ages 16 to 35 years old who planned to start or cease using HCs during the study. Controls were women who were not using a HC method. Eligibility criteria included not currently being pregnant, having a uterus (not post-hysterectomy), and not having any implanted uterine devices (such as Mirena or Paraguard). In addition, women diagnosed with illnesses that alter their immune system or hormone levels or who take medications that cause changes in their immune system or hormone levels were not eligible. Between 2011 and 2016, the HCL Study enrolled 125 women in the Baltimore, Maryland area.

In 8 study visits over 2 years, participants provided blood and urine samples, completed medical history and behavior questionnaires, and collected cervical and vaginal swabs. Visits were scheduled at baseline, 2 and 4 weeks, 3, 6, 12, 18, and 24 months. In addition, participants self-collected mid-vaginal swabs twice-weekly in the two weeks prior to each visit. HCL participants submitted over 4,500 vaginal samples. Collaborators at the University of Maryland School of Medicine Institute for Genome Sciences (UMSOM IGS, Brotman laboratory) analyzed these samples by 16S rRNA gene amplicon sequencing, and the data were merged with responses to the comprehensive reproductive health surveys.

The specific variables obtained in the Parent study questionnaires and clinical exams that were used in the current analysis were chosen based on literature reviews. A list of these variables and the HCL questionnaires that were obtained are included in Table 1.

Table 1: Hormonal Contraceptive Longitudinal Study (HCL, parent study) questionnaires and selected variables

Questionnaire Name	Selected Variables Included
Demographics	Age, race/ethnicity, education, household income, income sources, and marital status
Health and Medical History	Antibiotic use
Oral Health and Activity	Cigarette smoking
Reproductive Health and Activity	Self-reported BV diagnosis, douching, lubricant use, sexual partners, hormonal contraceptive use, menstrual sanitary and feminine hygiene product use
Clinical Evaluation	Height, weight, Amsel criteria for the diagnosis of BV (pH, discharge consistency, presence of clue cells, whiff tests results), vaginal symptoms

HUMAN SUBJECTS

Prior to conducting this research, I completed the online Collaborative Institutional Training Initiative (CITI) human participants online training program as well as HIPAA training 125/201. Our team received Human Subjects Research approval from the Institutional Review Boards (IRBs) from University of Maryland, College Park (999286-1) and Baltimore (HP-00073692). In addition, Dr. Ghanem received IRB approval from the Johns Hopkins University IRB (NA_00043112/CIR00024424) to transfer contact information of former HCL participants. All IRB approval letters are included in **Appendix A**. The research team affirmed that participant confidentiality and privacy would be maintained.

DATA COLLECTION

After receiving IRB approval from all sites, HCL study staff transmitted contact information of participants who agreed to future contact. As part of the HCL Study informed consent and privacy authorization process, participants could decline consent for participation in future research studies. Only four members (3.2%) of the original cohort did not consent to be contacted by future researchers. I did not receive contact information for these women. For the remaining 121 participants, I received protected health information (PHI), including participant email addresses, phone numbers, and mailing addresses, as well as the link between the subjects' identification numbers and corresponding PHI. I was the only member of the team who had access to the PHI and stored this information in a password-protected laptop. The rest of the research team received de-identified data.

Over 4 weeks, former participants were contacted by phone and email. In cases where initial contact was made via email, I made arrangements to call the participant at a convenient time. Verbal informed consent was obtained from participants specifically for the current research (see oral consent form in **Appendix B**). They had the opportunity to ask questions and decline at any time. At the time of re-contact for the birth mode study, all participants were 18 years old or older. None of the women seemed unable to render informed consent.

After consent was received, I administered the 3-item questionnaire (**Appendix C**), in which participants self-reported their birth mode, age at first menstrual period, and perceived weight status at menarche. Three women seemed unsure about their answers initially, particularly their age at menarche, and took the opportunity to contact their mothers to confirm. I received confirmation from all of these women, and only one changed an answer she had previously given. Overall, participants were very confident in reporting their birth mode responses. Participants were informed that they would receive a check for $10 as compensation for their time. At the end of the calls, participants verified their current mailing addresses to receive the check. In several cases, women also informed me that their last names had changed. The call, including describing the study, obtaining consent, administering the survey, and confirming the current mailing address, took an average of 7 minutes.

While most women completed the survey via phone interview, six participants asked to receive the questionnaire online, instead. They cited privacy and lack of time to complete the call as reasons why they preferred the online version. The text of the online survey programmed in REDCap is identical to the script used in phone interviews. A

final screen in the online survey allowed participants to enter their full name and current address. REDCap was used to store the database with all responses to this survey, including those obtained via phone interview.

Our team has completed data collection for this project, and we will return the PHI file with annotations regarding disconnected phone numbers and email addresses to the researchers at Johns Hopkins. All copies of the PHI file retained at IGS will be deleted.

SPECIMEN COLLECTION AND ANALYSIS

This analysis relies on vaginal specimens previously collected by the HCL study researchers. While many members of the cohort gave vaginal swabs repeatedly over two years, this cross-sectional study will focus on a vaginal swab collected for each participant by a study clinician at the enrollment visit (baseline).

At IGS, processing of vaginal swabs included the extraction of bacterial DNA, polymerase chain reaction (PCR) amplification, and 16S rRNA gene amplicon sequencing of the V3-V4 hypervariable regions. Ravel and IGS collaborators developed protocols for DNA extraction and bacterial sequencing, which are described in more detail in published articles [18, 64]. PCR amplification was performed using dual barcoded primers targeting the 338F to 806R region and sequenced on the Illumina HiSeq platform. Pre-processing of sequencing reads included assembly of forward and reverse reads and the removal of chimeric sequences. Species-level taxonomic assignments of each sequence read were performed using a classifier algorithm called

Support Vector Machine, which was trained on over 4000 specimens. For each sample, vectors of phylotype proportions were clustered into CSTs using this algorithm. Initial sequencing work for these study samples was completed in August 2016. However, the IGS groups plans to release new classification data for HCL using a newly-developed classifier algorithm, PECAN, later this year [65].

In early analysis of the HCL sequences, seven CSTs were identified. As previously described, CSTs I, II, and V indicate dominance by *Lactobacillus crispatus*, *L. gasseri*, and *L. jensenii*, respectively. Based on previous findings related to pH and Gram staining, these CSTs are characterized as "healthy" or promoting stability of the microbiota [40]. The sequenced dataset included designations for CSTs III-A and III-B, both dominated by *L. iners*. The distinction between the sub-types are based on the other bacterial taxa found in the samples; CST III-B tends to have a more diverse assemblage of BV-associated anaerobes (*G. vaginalis*) and lower relative abundance of *L. iners* compared to III-A, which is dominated by *L. iners* and not BV-associated bacteria. Preliminary and unpublished research conducted by Ravel and colleagues suggests that CST III-B more strongly resembles the diversity of bacteria found in CST IV and consequently may be more highly associated with vaginal dysbiosis and BV. CST IV was also divided, where IV-A is characterized by anaerobic bacteria including *Anaerococcus* and *Prevotella* spp., and IV-B contains higher proportions of *Gardnerella, Atopobium* and *Megasphaera*. This analysis includes a model that combines both CST III sub-types with CST IV in the dysbiotic group and a separate model that clusters CST III-A with the other *Lactobacillus*-dominated groups (CSTs I, II, and V) and only includes CST III-B and CST IV in the dysbiotic group.

We discovered that two participants, E020 and E082, submitted baseline vaginal samples that had less than 2,700 bacterial reads and were classified as CST I. In reviewing their subsequent samples submitted at least twice per week for 3 months, their samples were consistently classified as CST IV for E020 and CST III for E082 with much higher total bacterial reads. The enrollment sample for these participants was dropped from the analysis and replaced with a vaginal sample collected 2 days later, which in both cases had a sufficient number of bacterial reads such that it could be used for analysis.

STATISTICAL ANALYSIS

Data analysis involved modeling using STATA 10.0 for Windows (Stata Corporation, College Station, Texas) and SAS 9.13 (SAS Institute, Cary, NC). Descriptive analyses of the analytic sample were reported for key demographic variables. For each categorical demographic variable, a Fisher's exact test was used to test the association with birth mode because there were factors that had a few observations in each stratum.

Aim 1 tested the association between vaginal microbial composition and birth mode in this sample. Logistic regression was used to estimate odds ratios (ORs) with 95% confidence intervals (CIs) for several models. To compensate for the small sample size and limited power, we created binary variables from variables of interest. For instance, the CST variable was made binary by grouping *Lactobacillus*-dominant (CSTs I, II, III, and V) types in contrast with *Lactobacillus*-diminished CST IV. Other binary categorizations of CST were also explored, including CST III and IV sub-types. In addition, we created a dummy variable for race grouping African American and Hispanic

(AAH) women separately, due to low Latina representation in the sample as well as support in the literature for similar microbiota outcomes between the two racial/ethnic categories [18]. We included unadjusted models and considered models adjusted for confounding factors, including douching and education.

For Aim 2, we tested for effect modification by race/ethnicity in the association between birth mode and CST. Again, race/ethnicity was collapsed into a binary variable contrasting AAH and non-AAH women. We used a stratified regression approach and an interaction term approach to investigate potential effect modification [66]. Aim 3 dealt with the role of childhood factors—age and weight status at menarche—in the model. Both variables were evaluated as potential confounders, and models with adjustment for these childhood factors were compared to models with adjusted for douching status and education.

To visualize differences in the bacterial communities between women born vaginally and via C-section, we developed a heatmap in R (R Foundation for Statistical Computing, Vienna, Austria). The heatmap included the 25 most abundant bacterial taxa found in the samples and were color-coded based on the percent abundance of each bacterial type in the sample.

CHAPTER III: RESULTS

We recruited 88 women to answer our questionnaire, equaling 70.4% of the full HCL cohort and 72.7% of the participants who consented to future contact (**Figure 2**). Roughly 18% (n=16) of these women were born via C-section. The mean age of the study participants at HCL study enrollment was 26.2 (SD: 4.4; range: 17.4-34.6). Women were interviewed for the birth mode sub-study between 1 and 6 years after their enrollment in the parent study. The women represented seven self-reported racial/ethnic groups: Asian/Pacific Islander (n=2), Black/African American (n=29), Hispanic/Latina (n=1), Native American (n=1), White/Caucasian (n=48), mixed race (n=5), and other (n=2). The average age at first menstrual period was 12.0 (SD: 1.76; range 8-17). Most women responded that they were average weight at menarche (n=63, 71.6%). Approximately 9% responded that they were some degree of underweight (n=8) and over 18% were some degree of overweight (n=16) at the time of menarche. The majority of participants (56%) provided vaginal samples characterized as CSTs I (n=43), II (n=1), and V (n=5), which indicate dominance by *L. crispatus, L. gasseri*, and *L. jensenni*, respectively. Several participants' (25%) vaginal microbiota were classified as III-A (n=10) or III-B (n=12), indicating dominance by *L. iners*. Finally, approximately 19% of participants had vaginal microbiota with low abundance of *Lactobacillus* spp. and were classified as IV-A (n=7) or IV-B (n=10).

The heatmap in **Figure 3** describes the bacterial community composition of the vaginal microbiota of the study population. The data are sorted first by the birth mode of the participant and then by CST. Each column represents the vaginal microbiota sampled

from a woman at a single time point. The proportions of the 21 most abundant bacterial taxa within each sample are indicated by the legend in the top left.

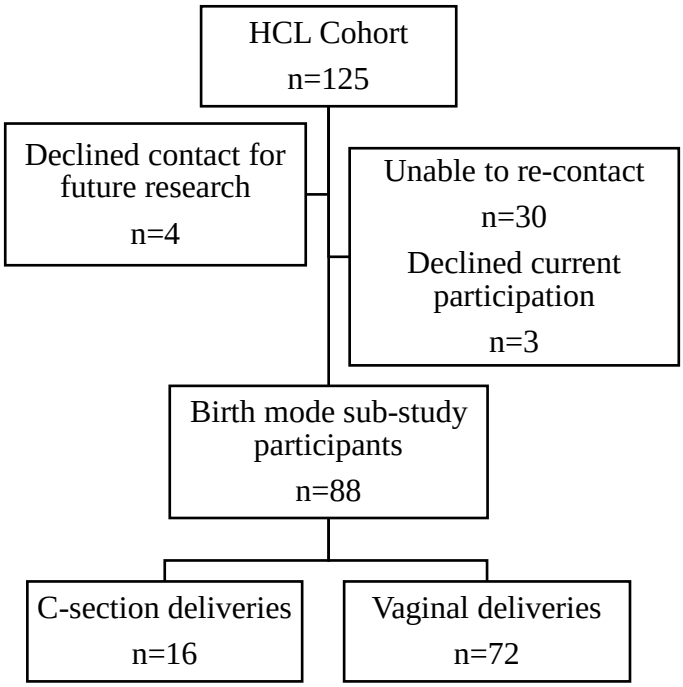


Figure 2: Birth mode study recruitment and participation

Table 2 outlines the demographics of the population tabulated by mode of birth. The groups born via C-section and vaginal delivery were similar demographically; Fisher's exact tests for most demographic factors yielded statistically insignificant differences. However, women born via C-section appeared more likely to be CST IV, as opposed to CST I, II, III, and V (p=.01). There were some moderately significant findings, such as a trend indicating that women born via C-section had more years of education (p=0.15). In addition, C-section-born women were more likely to report a younger age at menarche (p=0.15), past douching (p=0.08), and tampon use at last menstrual period (0.11). The women who reported douching were more likely to be AAH

compared to other races ($p<0.01$). Of the three women who were clinically diagnosed with BV at baseline, two were CST IV, and one was categorized to CST III.

The results of several adjusted and unadjusted models for factors associated with C-section delivery are outlined in **Table 3**. Confounding factors included in the final adjusted models were past douching and education, covariates that were found via Fisher's exact tests to be moderately associated with birth mode and CST. Overall, CST was significantly associated with birth mode, based on univariable and multivariable logistic regression modeling. Model 1 analyzed CST as a binary variable contrasting *Lactobacillus*-dominated vs. *Lactobacillu*s-deficient types and measured its association with the C-section outcome. Logistic regression indicated that women with a Lactobacillus-deficient CST IV were three times as likely to have a history of C-section (crude OR= 4.82, p=0.01, 95% CI: 1.46-15.88; adjusted OR= 3.73 (p=0.01, 95% CI: 1.08-12.77). In Model 2, vaginal microbiota was clustered into 3 broad groups. CSTs II, and V were included in the reference group with CST I, due to low representation of *L. gasseri*- and *L. jensenii*-dominant participants, and CSTs III and IV were analyzed separately. In this model, CST III was not associated with significantly increased odds of C-section history (adjusted OR=1.10, p=0.91, 95% CI: 0.29-5.30); however, CST IV maintained its significant association with C-section history compared to the reference group (OR=3.84, p=0.04, 95% CI: 4.01-14.56). BMI, race/ethnicity, age at menarche, BV diagnosis, perceived weight at menarche, and pH were found not to be associated with birth mode.

Based on two analytic approaches for determining effect modification (stratification and modeling with effect modification), there was some evidence (though

not overall statistically significant) that race modifies the relationship between vaginal CST and birth mode. All models grouped African American and Hispanic women (AAH) in contrast with other racial/ethnic groups. The results of the regression analyses stratified by race suggested that AAH who were CST IV were 8-fold more likely to have a C-section history (OR=8.89, p=.02) and among non-AAH women who were CST IV, the OR was 3-fold (OR=3.00, p=.18). However, a large p-value for the Breslow-Day test (0.393) indicated no significant difference in the odds ratios for the two racial/ethnic categories. Furthermore, a regression model with an interaction term for race and CST revealed a p-value which was not statistically significant (p=0.27).

To determine whether age and weight status at menarche had a profound confounding effect on the relationship between CST and birth mode, we adjusted Model 1 (which contrasted *Lactobacillus*-dominated vs *Lactobacillus*-deficient CSTs) by each of these puberty factors. After adjustment for age at menarche (as a continuous variable), women with Lactobacillus-deficient microbiota had a 4-fold increased likelihood of C-section history (OR=4.75, p=0.01, 95% CI: 1.44-15.65). Adjustment for self-reported weight status at menarche grouped as normal to underweight versus overweight and markedly overweight generated an OR of 4.82 (p=0.01, 95% CI: 1.463-15.89). Both adjusted models produced a change in the crude OR that was less than 10%, leading us to conclude that these puberty factors do not have a significant confounding effect on the relationship between CST and birth mode.

Figure 3: Heatmap of bacterial relative abundances from 88 samples

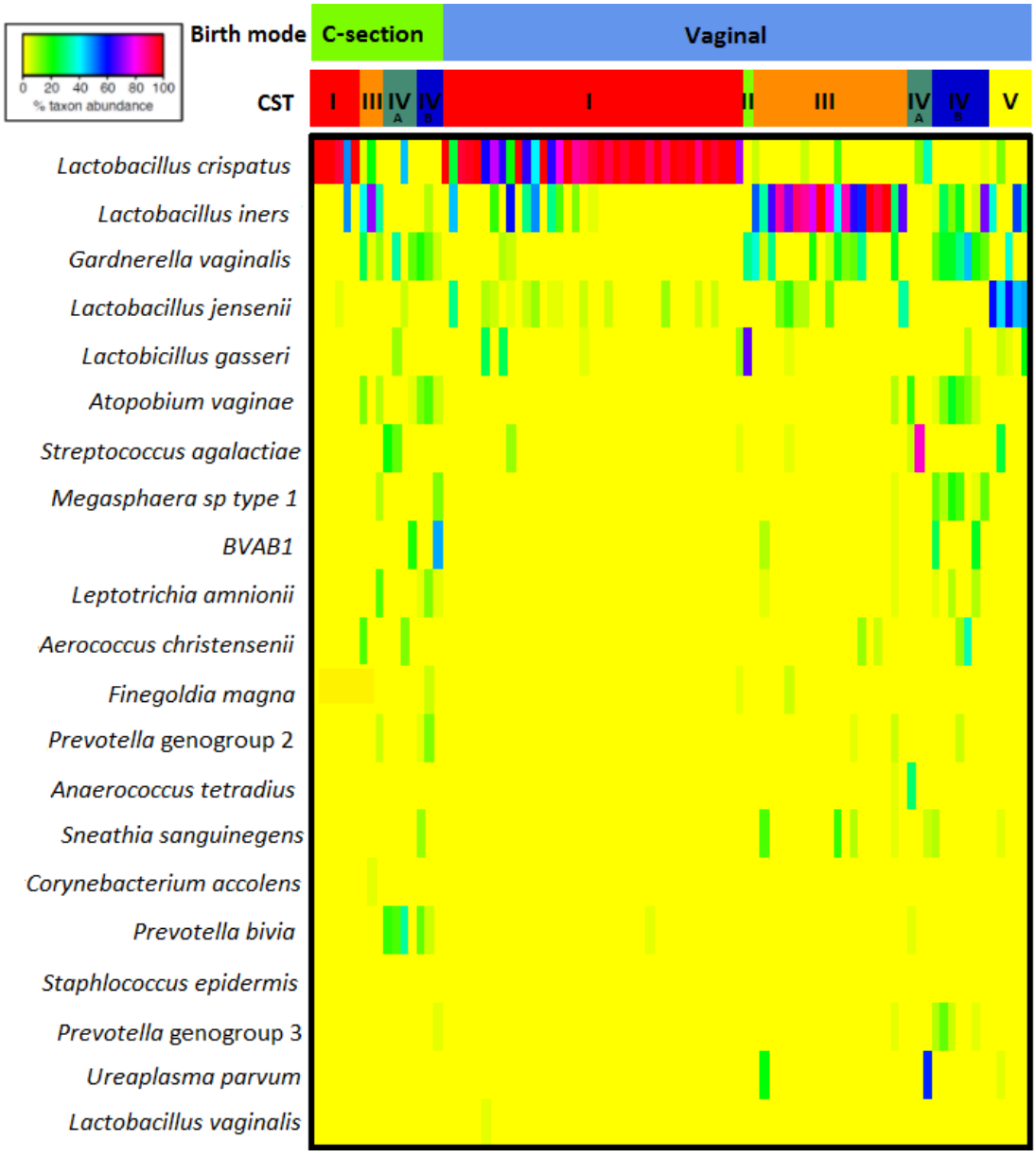


Table 2. Characteristics of birth mode sub-study participants (n=88).

		C-Section (n=16)		Vaginal (n=72)		
		n	%	n	%	p-value*
DEMOGRAPHICS	**Race/ethnicity**					0.39
	Asian/ Pacific Islander	0	0.0	2	2.8	
	Black/ African American	6	37.5	23	31.9	
	Hispanic/ Latina	1	6.3	0	0.0	
	White	8	50.0	40	55.6	
	Other	1	6.3	7	9.7	
						0.27
	Black or Latina	9	56.3	49	68.1	
	non-Black and non-Latina	7	43.8	23	31.9	
	Age at HCL parent study entry					0.44
	17 to 23	7	43.8	27	37.5	
	24 to 30	8	50.0	30	41.7	
	31 & over	1	6.3	15	20.8	
	BMI [a]					0.14
	<24.9	9	56.3	33	45.8	
	24.9-29.9	0	0.0	14	19.4	
	>29.9	7	43.8	25	43.8	
						0.32
	<24.9	9	56.3	33	45.8	
	>24.9	7	43.8	39	54.2	

Table 2. Characteristics of birth mode sub-study participants (n=88).

	C-Section (n=16)		Vaginal (n=72)		
	n	%	n	%	p-value*
Education					0.15
up to high school	0	0.0	9	12.5	
some college and above	16	100.0	63	87.5	
Income					0.52
less than $3000 per month	10	62.5	40	55.6	
$3001 or more per month	5	31.3	18	25.0	
no answer	1	6.3	14	19.4	
Income source					0.44
job	9	56.3	40	55.6	
significant other, family, or friends	0	0.0	9	12.5	
government assistance	1	6.3	3	4.2	
multiple	6	37.5	20	27.8	
Marital Status					0.23
Never married	11	68.8	61	84.7	
Married	4	25.0	7	9.7	
Divorced	1	6.3	2	2.8	
Other	0	0.0	2	2.8	

Table 2. Characteristics of birth mode sub-study participants (n=88).

			C-Section (n=16)		Vaginal (n=72)		
			n	%	n	%	p-value*
CLINICAL CHARACTERISTICS	**CST**						0.12
	I	(*L. crispatus*-dominated)	6	37.5	37	51.4	
	II	(*L. gasseri*-dominated)	0	0.0	1	1.4	
	III	(*L. iners*-dominated)	3	18.8	19	26.4	
	IV	(Low-*Lactobacillus*)	7	43.8	10	13.9	
	V	(*L. jensenni*-dominated)	0	0.0	5	6.9	
							0.09
	CST I, II, V		6	37.5	43	59.7	
	CST III, IV		10	62.5	29	40.3	
							0.01
	CST I, II, III, V		9	56.3	62	86.1	
	CST IV		7	43.8	10	13.9	
	BV diagnosed within 2 months**						0.45
	no BV		15	93.7	70	97.2	
	BV		1	6.2	2	2.8	
	Vaginal pH						0.40
	4.0-4.5		10	62.5	51	73.9	
	4.6-5.0		2	12.5	10	14.5	
	>5.0		4	25.0	8	11.6	

Table 2. Characteristics of birth mode sub-study participants (n=88).

	C-Section (n=16)		Vaginal (n=72)		
	n	%	n	%	p-value*
Vaginal pH (continued)					0.27
4.0-4.5	10	62.5	51	73.9	
>4.5	6	37.5	18	26.9	
Vaginal signs (self-reported at clinical visit)					
Discharge	1	6.3	2	2.8	0.46
Itching	1	6.3	0	0.0	0.18
Ever been pregnant					0.61
no	10	62.5	45	62.5	
yes	6	37.5	27	37.5	
Ever given birth vaginally					0.58
no	13	81.3	57	79.2	
yes	3	18.8	15	20.8	
Ever given birth by C-section					0.52
no	14	87.5	65	90.3	
yes	2	12.5	7	9.7	
Years since last pregnancy					0.51
none	11	68.8	45	62.5	
1	2	12.5	5	6.9	
2+	3	18.75	22	30.6	

Table 2. Characteristics of birth mode sub-study participants (n=88).

		C-Section (n=16)		Vaginal (n=72)		
		n	%	n	%	p-value*
PUBERTY	**Age at menarche**					0.17
	≤11	6	42.9	30	41.7	
	12	6	37.5	11	15.3	
	13	1	6.3	17	23.6	
	≥14	3	18.8	14	19.4	
						0.15
	≤12	12	75.0	41	56.9	
	>12	4	25.0	31	43.1	
	Weight status at menarche [a]					0.61
	Average or below	13	81.3	58	81.7	
	Overweight or above	3	18.8	13	18.3	
SEXUAL HEALTH AND BEHAVIOR	**Hormonal contraceptive use (current)**					0.43
	no	9	56.3	36	50.0	
	yes	7	43.8	36	50.0	
	Number of sexual partners in the prior 2 months [b]					0.24
	None	4	28.6	5	8.6	
	1	10	71.4	51	87.9	
	2+	0	0.0	2	3.5	
						0.20
	none	4	25.0	10	14.3	
	1+	12	75.0	60	85.7	

Table 2. Characteristics of birth mode sub-study participants (n=88).

		C-Section (n=16)		Vaginal (n=72)		
		n	%	n	%	p-value*
OTHER HEALTH	**Douched (ever)**					0.08
	no	9	56.3	56	77.8	
	yes	7	43.8	16	22.2	
	Hygiene product use (2 months)					
	Feminine towelette	1	7.1	6	10.0	0.60
	Hygiene spray	0	0.0	3	5.0	0.53
	Hygiene powder	1	7.1	2	3.3	0.47
	Vaginal acid gel	1	7.1	0	0.0	0.18
	Sanitary product use at last menstrual period					0.23
	Tampon only	6	37.5	29	40.3	
	Sanitary napkin only	1	6.3	17	23.6	
	Tampon and sanitary napkin	9	56.3	26	36.1	
	Antibiotic use* **					0.62
	no	14	87.5	62	86.1	
	yes	2	12.5	10	13.9	
	Smoking					0.45
	no	14	87.5	66	91.7	
	yes	2	14.3	6	8.3	

*Reported p-values from Fisher's exact tests
**Includes clinician- and self-reported diagnoses
***Includes antibiotics used 2 months prior to (NOT prescribed at) enrollment visit

[a] Missing for 1 respondent
[b] Missing for 2 respondents
[c] Missing for 3 respondents

Table 3. Crude and adjusted odds ratios (ORs) and 95% confidence intervals (CIs) for factors associated with C-section delivery (n=88).

				Unadjusted			Adjusted*		
				OR	95% CI	p-value	OR	95% CI	p-value
	CST								
Model 1		I, II, III, V	(n=71)	REF			REF		
		IV	(n=17)	4.82	1.46-15.89	0.01	3.73	1.08-12.77	0.01
	CST								
Model 2		I, II, V	(n=49)	REF			REF		
		III	(n=22)	1.13	0.26-5.01	0.87	1.10	0.29-5.30	0.91
		IV	(n=17)	5.02	1.38-18.21	0.01	3.84	4.01-14.56	0.04
	BMI								
Model 3		<24.9	(n=42)	REF			REF		
		>=24.9	(n=46)	0.66	0.22-1.96	0.45	0.57	0.08-1.35	0.12
	Race								
Model 4		All other races	(n=58)	REF			REF		
		Black and Hispanic	(n=30)	1.66	0.55-5.00	0.37	1.50	0.44-5.14	0.52
Model 5	**Age at menarche**		(continuous, n=88)	1.01	0.74-1.37	0.97	0.93	0.64-1.28	0.56
Model 6	**Clinical BV diagnosis**		(n=3)	0.89	0.10-8.22	0.92	1.75	0.31-16.75	0.73

Table 3. Crude and adjusted odds ratios (ORs) and 95% confidence intervals (CIs) for factors associated with C-section delivery (n=88).

			Unadjusted			Adjusted*		
			OR	95% CI	p-value	OR	95% CI	p-value
	Perceived weight at menarche							
Model 7	overweight or markedly overweight	(n=16)	REF			REF		
	normal or underweight	(n=17)	0.97	0.24-3.91	0.97	1.56	0.39-5.42	0.72
	pH							
Model 8	4.0-4.5	(n=61)	REF			REF		
	>4.5	(n=24)	1.70	0.54-5.35	0.36	1.71	0.22-6.27	0.45

* All adjusted models were adjusted for douching and education.

CHAPTER IV: DISCUSSION

In this sample of 88 reproductive-age women, we found evidence to suggest that a woman's vaginal microbiota in adulthood is associated with her mode of birth history. Specifically, women whose vaginal microbiota was characterized as the low-*Lactobacillus* CST IV had a statistically significant 3-fold increase in the odds of being born via C-section. This indicates that women born by C-section were more likely to have a vaginal state characterized by higher proportions of BV-associated bacteria and higher likelihood for vaginal dysbiosis in adulthood [67]. BV remains a condition that is highly recurrent, associated with a number of adverse reproductive health outcomes and treatment and prevention remains remarkably ineffective [68]. This birth mode research may represent a potentially modifiable risk factor. Broadly, interventions may include reducing C-section rates, reseeding the infant girl microbiome with direct transfer from the mother, or probiotic cocktails administered at various phases of childhood. If such interventions have an effect on BV and vaginal dysbiosis, we will then have conclusive evidence on the causal link between the two.

We anticipated that race would be a confounding factor in the model, as literature suggests it is highly associated with both birth mode and CST outcomes. However, we found that race was not significantly associated with birth mode in this relatively small study. Furthermore, adjusting for race did not change the OR in Model 1 by 10% or more, leading us to drop the variable as a potential confounder. We also considered the confounding effect of several factors that encompass socioeconomic status (SES). Studies have shown that the chance of women undergoing C-section is associated with higher education, lower residential crowding, and pregnancy planning [69]. While education in

this study was only moderately associated with birth mode (p=0.15), it produced a significant confounding effect (14.5% change in OR). As 17% of participants chose not to respond to education questions at the enrollment visit, it was not a variable that could reliably be included in the model. Finally, douching behavior was moderately associated with birth mode (p=.08) but resulted in small confounding effect (5% change in ORs).

Women with a BV diagnosis were more likely to have a CST III or IV vaginal microbiota. However, BV was not specifically found to be associated with birth mode, as there were very few self- or clinician-reported cases of BV in the population (n=6). Half of these cases were self-reported, and self-reported BV is very unreliable, particularly since the condition can be asymptomatic. Since there were only three clinician-reported BV events at the enrollment visit, it was not possible to conduct further analysis on this variable. Of the women who had a recent BV diagnosis, only two were CST IV-B. The remaining four represented CST I and III-B.

We were unable to conclusively demonstrate effect modification due to race in our study, perhaps due to small sample size and limited power. However, it was clear through stratified regression modelling that AAH women born via C-section were far more likely to be CST IV compared to non-AAH women born similarly, a finding that reaffirms our hypothesis derived from previous research [18].

Age at menarche was not associated with a particular CST or birth mode. In addition, age and weight status at menarche did not produce a confounding effect greater than 10% on the relationship between CST and birth mode. This indicates that a woman's age or weight status at menarche did not distort the strong relationship between CST and birth mode in our population.

In summary, this study found significant 3-fold association between low-*Lactobacillus* microbial community state and being born via C-section, which indicates that C-section delivery may be related to vaginal dysbiosis in adulthood. Of the covariates we analyzed, factors of puberty were not shown to alter the strength of the relationship between adulthood CST and birth mode. Although AAH who were CST IV were more likely to have a history of C-section birth, race/ethnicity was not found to be an effect modifier. Although this study does not establish causality or characterize the long-term composition of the vaginal microbiota, results suggest that birth mode potentially impacts vaginal seeding and long-term disease outcomes.

STRENGTHS AND LIMITATIONS

CST was determined from one vaginal sample for each woman collected at the Parent study baseline visit. However, the vaginal microbiome is dynamic for many women and can change at various points in the menstrual cycle. Basing the overall CST assignment on one sampling day may result in misclassification. Given the rapid fluctuations in vaginal microbiota for many women, a cross-sectional study is limited in its ability to make conclusions about the composition and stability of the vaginal microbiota. The HCL dataset is longitudinal, with multiple samples taken over each participant's enrollment. A future related study can develop a "community class" that assess overall CST profile of each woman over many sampling days. In addition, Jensen-Shannon distances between all pairs of samples can assess the stability of the microbiota longitudinally. While the microbiota can be dynamic, longitudinal studies have also demonstrated that women tend to stay in the same plane of fluctuation over many months

and years of observation [40]. Women who are found in CST IV tend to fluctuate between IV and the *L. iners*-dominated CST III most often [70]. In addition, from an epidemiologic perspective, if there were non-differential misclassification of CSTs, that would serve to dampen the point estimates toward the null. That we found women with CST IV microbiotas had 3-fold statistically significant increased odds for C-section history indicates the association between birth mode and CST may be even more pronounced if we had more temporal observation points.

For two participants, vaginal sample reads from the baseline were replaced by a follow-up sample taken two days later due to low bacterial reads in the baseline sample. In both of these cases, it was clear that the baseline read was an aberration, and the microbiota classification for subsequent sample days remained very consistent. However, we did not measure the consistency of all participants' microbiota composition in this analysis.

Although the survey was very short, the questions themselves may be affected by recall bias. Surprisingly, most women seemed very sure about their own modes of birth. Several participants remarked that their mothers had told them the stories of their own birth many times and were fully aware of their status. The question on age at menarche caused more women to pause. Many were initially unsure of the exact age and pointed to a year in school (e.g., early in the fifth grade) in order to deduce their response. Participants were given the opportunity to reach out to their mothers or other family members to confirm their answers. Three women took this opportunity to confirm their age at menarche with a family member. All three of these women returned contact with us, and only one changed an answer she had previously given. It is still likely that these

birth and early-life factors will be reported with some error, given that the recall is many years later. In addition, self-characterization of childhood weight status is subjective. It appears that within this sample of young women, self-report of mode of birth was reliable; however, age at menarche and body mass in childhood was more difficult for adults to self-report.

A strength of this study is that we were able to recruit a sufficient number of women born via C-section. The proportion of the women born via C-section in the study (18%) reflects the estimated rates of C-section deliveries when the young adult participants were born. Between 1975 and 1990, the percentage of C-section deliveries in the United States rose from 10.4% to 24.5% [71]. In the past three decades, the effect of C-section on the infant microbiota has incurred greater scrutiny due to the increased rate of cesarean deliveries worldwide and their potential association with disease, making this research timely.

The study population was diverse, with women representing many race/ethnic groups, age groups, and other demographic categories. The HCL study aimed to recruit diverse participants in order to maximize generalizability of results. However, many of the women were students and employees at Johns Hopkins and other local universities, and consequently, the population was highly educated. The vast majority (89%) had some post-high school education with 47% having earned a bachelor's degree or higher. In contrast, 30% of Baltimore city residents over age 25 had a bachelor's degree or higher, according to 2011-2015 Census figures [72]. We also found that the study participants had a moderately high income status, with 26% reporting monthly earnings more than $3000. Although the group was highly educated, many women were between 17 and 23

years old, potentially explaining why the observed income status was not higher. Greater education in the population may improve access to care and decrease potential for BV.

Overall, the study design to re-contact HCL participants was successful, and we were able to reengage over 72% of the women who consented to be contacted for future research. Re-contacting participants took several weeks to complete. Many participant phone numbers and email addresses had been disconnected in the time since their involvement with HCL. However, many former participants who we were able to contact seemed enthusiastic to take part in the follow-up research. Self-selection bias was a minor concern, as women who participated in multiple follow-ups for the parent study have proven to be very motivated and may be different from women who refused to participate. The $10 check incentive was an important factor in encouraging women to participate in the survey. After the call several women expressed interested in seeing published results of the HCL study as well as participating in research in the future.

AREAS FOR FUTURE RESEARCH

We aim to include more diverse groups of women to participate in this birth mode research to increase power and potentially improve the generalizability of our results. Our research group has identified another concluded study with hundreds of women already enrolled who we could re-contact to answer the birth mode survey. Vaginal samples have already been collected and characterized at IGS. However, similar limitations still persist. This cross-sectional study does not allow for characterization of the microbiome over the life span, although it would be exceptionally costly to follow baby girls from birth to adulthood in a longitudinal study.

Future research into the effect of birth mode should also collect data on whether the C-section was an elective or emergency procedure. The reasons why C-section deliveries are performed vary by the complications of pregnancy or delivery that preceded the cesarean. It is possible that these underlying causes are more important than the C-section event itself in determining bacterial transfer between mother and neonate. When doctors recommend emergent C-section, it is often accompanied by the varying use of medications, including antibiotics and anti-inflammatory pain analgesics, which can have independent effects on the microbiome of mother and neonate. In addition, if the mother is experiencing obstetric distress at the time of pregnancy, the underlying cause could influence her microbiome and transmission to the neonate. For example, a mother's BV at birth is associated with adverse neonatal outcomes [73]. The effects of these birth factors are not well known, and therefore, it is important to note these factors in determining the protectiveness of either birth mode.

In addition, future researchers could analyze the enduring effects of interventions to seed babies who are delivered by C-section. This is a controversial intervention, and its long-term effects are not well understood. An ongoing clinical trial by Dominguez-Bello *et al*. (ClinicalTrials.gov Identifier: NCT02407184) seeks to understand whether the microbiota of infants born via scheduled C-section can potentially be restored via seeding when assessed at one year [74]. Our adulthood microbiota data, as well as studies assessing adolescent outcomes, may encourage researchers to schedule follow-ups as the infant ages and establish the first long-term studies on the effect of birth mode and human microbiome outcomes.

APPENDICES

APPENDIX A: INSTITUTIONAL REVIEW BOARD APPROVAL LETTERS

1204 Marie Mount Hall
College Park, MD 20742-5125
TEL 301.405.4212
FAX 301.314.1475
irb@umd.edu
www.umresearch.umd.edu/IRB

DATE: January 20, 2017
TO: Christina Stennett, BS, MPH Candidate
FROM: University of Maryland College Park (UMCP) IRB

PROJECT TITLE: [999286-1] Mode of birth and vaginal microbiota in non-pregnant reproductive-age women

REFERENCE #:
SUBMISSION TYPE: New Project

ACTION: APPROVED
APPROVAL DATE: January 20, 2017
EXPIRATION DATE: January 19, 2018
REVIEW TYPE: Expedited Review
REVIEW CATEGORY: Expedited review category # *5 and 7*

Thank you for your submission of New Project materials for this project. The University of Maryland College Park (UMCP) IRB has APPROVED your submission. This approval is based on an appropriate risk/benefit ratio and a project design wherein the risks have been minimized. All research must be conducted in accordance with this approved submission.

Prior to submission to the IRB Office, this project received scientific review from the departmental IRB Liaison. This submission has received Expedited Review based on the applicable federal regulations.

This project has been determined to be a Minimal Risk project. Based on the risks, this project requires continuing review by this committee on an annual basis. Please use the appropriate forms for this procedure. Your documentation for continuing review must be received with sufficient time for review and continued approval before the expiration date of January 19, 2018.

Please remember that informed consent is a process beginning with a description of the project and insurance of participant understanding followed by a signed consent form. Informed consent must continue throughout the project via a dialogue between the researcher and research participant. Unless a consent waiver or alteration has been approved, Federal regulations require that each participant receives a copy of the consent document.

Please note that any revision to previously approved materials must be approved by this committee prior to initiation. Please use the appropriate revision forms for this procedure.

All UNANTICIPATED PROBLEMS involving risks to subjects or others (UPIRSOs) and SERIOUS and UNEXPECTED adverse events must be reported promptly to this office. Please use the appropriate reporting forms for this procedure. All FDA and sponsor reporting requirements should also be followed. All NON-COMPLIANCE issues or COMPLAINTS regarding this project must be reported promptly to this office.

Please note that all research records must be retained for a minimum of seven years after the completion of the project.

If you have any questions, please contact the IRB Office at 301-405-4212 or irb@umd.edu. Please include your project title and reference number in all correspondence with this committee.

This letter has been electronically signed in accordance with all applicable regulations, and a copy is retained within University of Maryland College Park (UMCP) IRB's records.

JOHNS HOPKINS
MEDICINE

Office of Human Subjects Research
Institutional Review Boards

1620 McElderry Street, Reed Hall, Suite B-130
Baltimore, Maryland 21205-1911
410-955-3008
410-955-4367 Fax
e-mail: jhmirb@jhmi.edu

Date: February 17, 2017

CHANGE IN RESEARCH APPROVAL

Review Type:	Convened
Principal Investigator:	Khalil Ghanem
Number:	NA_00043112 / CIR00024424
Title:	Duration of Hormonal Contraceptive Use: Immune Responses & Vaginal Microbiota
Committee Chair:	Joseph Carrese
IRB Committee:	IRB-5

Date of approval: February 13, 2017

Date of Expiration: March 27, 2017

The JHM IRB approved the above-referenced Change In Research.

Chang ein research is approved. Approval includes the following:

1 revised eForm A protocol (dated 2/6/2017)
1 revised oral consent script
1 new supplemental study document.

IRB review included the following:

Use of an oral consent process.

Study Team Members:
Rebecca Brotman, Anne Burke, Roxanne Jamshidi, Patti Gravitt, Rupak Shivakoti, Barbara Wilgus, Sabra Klein, Susan Tuddenham, Johan Melendez, Catherine Murphy, Linda Rogers, Jean Anderson, Barbara Detrick, Kathryn Chang, Stephanie Nasatka, Stephanie McLaughlin, Jonathan Zenilman, Sam Wilgus, Rebecca Ozl, Denice Jones

From: HRPO Cicero
To: Stennett, Christina
Subject: External IRB Record Activity
Date: Friday, February 24, 2017 11:44:31 AM

Notification of Use of An External IRB

PI: Rebecca Brotman

Study Number: HP-00073692

Study Name: Mode of birth and vaginal microbiota

Date: 1/20/2017

You have indicated that you will be using an IRB external to UMB for review and oversight of this research project. Investigators are responsible for being knowledgeable of and compliant with all UMB HRPP policies described in the HRPP Plan and Investigator Manual found at http://www.hrpo.umaryland.edu/researchers/investigator_manual.html.

APPENDIX B: ORAL CONSENT SCRIPT

Date: January 30, 2017
Principal Investigator: Rebecca Brotman, PhD
Application No.:

ORAL CONSENT SCRIPT

Protocol Title: Mode of birth and vaginal microbiota in reproductive-age women

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Hello, my name is Christina Stennett, and I am a graduate student at the University of Maryland. I am conducting a research study with women who participated in the Johns Hopkins Hormonal Contraceptive Longitudinal Study to understand how your mode of birth (whether by C-section or vaginal delivery) may affect your gynecologic health as an adult. As a former HCL Study participant, you are being asked to take part in this research study.

If you agree to participate, I will ask you questions about your birth and childhood. This will take less than 10 minutes of your time. We appreciate you taking the time to participate in this study and understand your time is precious. Therefore, we you will receive a $10 check as a token of our appreciation for your participation.

We will minimize any losses to confidentiality or privacy due to your participation. Your responses will be kept confidential and will be stored in password-protected computers and files. Your answers will be grouped with information from other participants in any published manuscripts.

There is no direct benefit to you from participating. However, the findings will help researchers better understand how birth and early childhood factors affect vaginal health in adulthood.

You do not have to agree to be in this study. If you join the study, you can change your mind later. You can decide not to take part or you can quit at any time. There will be no penalty or loss of any benefits if you decide to quit the study. If you do not want to join the study, it will not affect your care at Johns Hopkins. You can choose not to answer individual questions, and if at any time you decide you do not wish to participate any further, you can decline further participation.

If you have any questions about your rights as a research participant, or if you think you have not been treated fairly, you may call the following Institutional Review Board (IRB) offices:

- University of Maryland College Park IRB Board Office at (301) 405-0678
- Johns Hopkins University IRB at 410-955-3008

People at Johns Hopkins who are involved in the study or who need to make sure the study is being done correctly will see the information. In addition, people at Johns Hopkins may need to send your information to people outside of Johns Hopkins (for example, government groups like the Food and Drug Administration) who need to make sure the study is being done correctly. These people will use your information for the purpose of the study.

We will continue to collect information about you until the end of the study unless you tell us that you have changed your mind. If you change your mind and don't want your information used for the study anymore, you can call IRB offices of University of Maryland College Park or Johns Hopkins University. Just remember, if we have already used your information for the study, the use of that information cannot be cancelled.

We try to make sure that everyone who needs to see your information uses it only for the study and keeps it confidential - but, we cannot guarantee this.

Do you have questions about the study?

If you agree to participate in this research, please say "I consent."

If you have questions about the study in the future, contact me at (410) 706-4252 or cstennett@som.umaryland.edu.

APPENDIX C: TELEPHONE QUESTIONNAIRE

Thank you for taking part in this study! The questionnaire is only 3 items long. Note that these questions are about your own birth and childhood (NOT your kids'). If you don't know or are unsure of any of the answers, please tell me.

A. Were you born via vaginal delivery or Cesarean section (C-section)?

- ☐ Vaginal
- ☐ C-section
- ☐ I don't know

  [Please feel free to contact your mother, other family members, or friends of your family to confirm.]
- ☐ Prefer not to answer

B. How old were you at your first menstrual period?

- ☐ Age: _____ (Record exact age or range given.)
- ☐ I don't know

  [Please feel free to contact your mother, other family members, or friends of your family to confirm.]
- ☐ Prefer not to answer

C. How would you classify your own weight at the time of your first menstrual period (around age 10-13)?

- ☐ Markedly underweight
- ☐ Underweight
- ☐ Average
- ☐ Overweight
- ☐ Markedly overweight
- ☐ I don't know

  [Please feel free to contact your mother, other family members, or friends of your family to confirm.]
- ☐ Prefer not to answer

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